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Recent Studies of the Enzymic Synthesis of Ricinoleic Acid by Developing Castor Beans[1]

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ABSTRACT

Oleate Δ^{12}-hydroxylase activity was measured in extracts of developing castor bean seeds. Most of the hydroxylase activity is associated with microsomes. However, when microsomes are washed, the activity is completely lost. Some (50%) of the activity can be restored by addition of the 100,000*g* supernatant to the washed microsomes. Supernatant extracts (100,000*g*) of developing safflower seeds are able to restore all (100%) of the hydroxylase activity to the washed castor bean microsomes. In addition, purified mammalian catalase can restore some (25%) of the activity to the microsomes but is not as effective as either castor bean or safflower 100,000*g* supernatants. The K_m of the hydroxylase for oxygen is 4 micromolar. Inasmuch as the activity was not inhibited by high concentrations of either carbon monoxide or cyanide, neither the involvement of cytochrome P450 nor other cytochrome systems is suggested. The enzyme system was not saturated by oleoyl-CoA, even at concentrations as high as 200 micromolar. When [^{14}C]oleoyl-CoA is supplied as a substrate, the acyl component is rapidly transferred to phosphatidylcholine (PC). Hydroxylation may occur on PC or on a lipid which receives its acyl component from PC. However, exogeneously added 2-[1-^{14}C]oleoyl-PC was hydroxylated at a much lower rate than was [1-^{14}C]oleoyl-CoA added as the primary substrate.

The *in vitro* synthesis of ricinoleic acid [D(+)-12-hydroxy-*cis*-9-octadecanoic acid] was partially characterized by Galliard and Stumpf in 1966 (6). They described the hydroxylation of oleoyl-CoA by a microsomal mixed-function oxidase which required molecular O_2 and NADH. Inasmuch as it has recently been shown in several plant tissues (12, 15, 16) that oleate desaturation actually occurs on PC[2] and not on oleoyl-CoA, as was previously believed (17), experiments were designed to elucidate the immediate substrate of oleate hydroxylation in castor bean and to compare the mechanism to that of oleate desaturation in other plants.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleoyl-CoA (47.7 mCi/mmol) was obtained from New England Nuclear. 2-[1-^{14}C]Oleoyl-phosphatidylcholine (5.23 mCi/mmol) was prepared by incubating 2 μCi [1-^{14}C]oleoyl-CoA with 1 ml 12,000*g* developing castor bean supernatant, and 200 μl 1.0 M Tris HCl buffer (pH 7.0), anaerobically for 5 min (see Table II for evidence that the PC was labeled at carbon 2). The reaction was stopped with 1 ml hot isopropanol and heated to 80 C for 10 min. Lipids were extracted and PC was separated by TLC as described below. The TLC spot containing PC was sequentially eluted with 2 ml 50 mM acetic acid, 3.3 ml ethanol, and 6.6 ml chloroform. ACP was isolated from *Escherichia coli* by the method of Majerus *et al.* (9). All other reagents were obtained from Sigma. Developing castor bean seeds (*Ricinus communis* var Baker 290) were harvested from the field from August to December, 1979.

Preparation of Cell Fractions. As mentioned previously (6), maximum hydroxylase activity was obtained during a narrow period of seed development. For the study reported here, seeds were chosen when their endosperm filled all but the outer 1 mm of the volume of the seed. A previous study showed that, at this stage, 80 to 90% of the storage lipid had been synthesized (11). After removal of the seed coat, the endosperm and embryonic axes were washed in cold water and homogenized with a mortar and pestle in 2 volumes grinding buffer (0.6 M sucrose, 0.15 M Tricine, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and the pH was adjusted to 7.5 with KOH). The homogenate was filtered through 44-μm nylon cloth and centrifuged at 500*g* for 10 min. The 500*g* supernatant and fat pad were combined and centrifuged at 12,000*g* for 30 min. To 2 ml 12,000*g* supernatant was added 0.02 volume 100 mM α-tocopherol (or other antioxidants when noted) in ethanol, and the preparation was centrifuged at 105,000*g* for 1 h. The 100,000*g* pellet (microsomes) was resuspended in 2 ml grinding buffer and 0.02 volumes 100 mM α-tocopherol with a Teflon homogenizer. The resuspended microsomes then were washed by recentrifugation at 105,000*g* for 1 h. This washed microsomal pellet was finally resuspended in 2 ml grinding buffer and 0.02 volume 100 mM α-tocopherol.

Enzyme Assays. Oleate hydroxylase was routinely assayed for 30 min at 30 C in screw-cap vials by a radioisotopic method. The reaction mixture contained 0.3 M sucrose, 0.1 M Tris-HCl buffer (pH 7.0), 0.2 mM NADH, 0.4 nmol [1-^{14}C]oleoyl-CoA (about 40,000 cpm), and 25 μl enzyme in a total volume of 1 ml. The reaction was stopped by adding 1 ml 15% methanolic KOH. The lipids were saponified by capping the vials and heating at 80 C for 30 min. The samples then were cooled and neutralized with HCl, and the lipids were extracted with 7 ml hexane:isopropanol (3:2) and 5 ml 6.7% Na_2SO_4. The fatty acids were methylated with diazomethane and separated on a stainless steel column (155 cm × 7 mm) packed with GP88-SE30 (14.3% on Chromosorb W, AW 60/80 mesh; Analabs). A Varian-Aerograph 920 gas chromatograph was used, coupled with a Nuclear-Chicago Biospan 4998 proportional radiation detector and a Nuclear-Chicago model 8770 integrator. At 220 C, methyl oleate and methyl ricinoleate have retention times of 3 and 9 min, respectively.

Catalase was assayed spectrophotometrically by measuring the decrease in H_2O_2 at 240 nm.

NADH oxidation was measured with an Aminco J4-7439 Fluoro-Colorimeter equipped with excitation filter 7-60 (360 nm maxima) and emission filter 2A (>410 nm). The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.0), 10 μM NADH, and 50 μl enzyme in a reaction mixture of 2 ml.

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[2] Abbreviations: PC, phosphatidylcholine; ACP, acyl carrier protein.

Acyltransferase activity was measured in a reaction mixture containing 0.3 M sucrose, 100 mM Tris-HCl (pH 7), 0.4 nmol [1-^{14}C]oleoyl-CoA (40,000 cpm), and 100 μl enzyme in a volume of 1 ml. After 10 min of incubation, the lipids were extracted with hexane-isopropanol as previously described. PC was separated from other lipids by TLC as described below. The PC spot was scraped and counted with Phase Combining System (Amersham)-xylene (2:1) (Beckman). Protein was measured by the Bradford method (4).

Lipid Class Analysis. When it was necessary to determine the amount of label in the various lipid classes, the lipids were extracted with chloroform-methanol according to the method of Bligh and Dyer (3). In an early experiment, samples of the aqueous phases of chloroform-methanol-extracted reaction mixtures incubated at various times were saponified, methylated, and run on radio-GLC to assay for acyl-CoA. Control experiments with known [^{14}C]oleoyl-CoA indicated counts in the aqueous phase to be associated only with the acyl-CoA. In subsequent experiments, radioactivity in the aqueous phase was assumed to represent only oleoyl-CoA. The lipids were separated on 250 μm Silica Gel G TLC plates by development with chloroform:methanol:acetic acid:H_2O (85:15:10:3.5, v/v). The R_F values for lyso-PC, PC, and nonpolar lipids were 0.03, 0.22, and 0.88, respectively. When necessary, the lipid spots were scraped, saponified, methylated, and analyzed as described above.

Oxygen and Carbon Monoxide Studies. Mixtures of gases were blended with a Wostoff model M 100 A Gas Mixing Pump (Calibrated Instruments, Ardsley, NY). The standard reaction mixture was prepared in modified Thunberg tubes. A vacuum was drawn, the tubes were flushed with gas mixture for 5 min and sealed, and the reactants then were combined and incubated for 30 min.

RESULTS AND DISCUSSION

Characteristics of Centrifugal Fractions. After centrifugation at 500*g* for 10 min or after 12,000*g* for 30 min, almost all of the total hydrolase activity was found in the 12,000*g* supernatant. Several sucrose density gradients of extracts of developing castor bean seeds were prepared (data not shown). Because there was a high level of breakage of proplastids as reported by others (10), there was no consistent pattern of hydroxylase activity in the gradients. Perhaps when the primary substrate of the oleate hydroxylase is identified and added to the various organelle fractions, it then will be possible to determine the precise subcellular localization of the hydroxylase.

When the 12,000*g* supernatant was centrifuged at 100,000*g* with α-tocopherol for 1 h, 29% of the activity was recovered in the pellet and only a small amount was found in the supernatant (Table I). The rest of the activity (about 65%) was lost during centrifugation. When α-tocopherol was omitted, all activity was lost during the 100,000*g* centrifugation as previously reported (6). Various other antioxidants (butylated hydroxytoluene and propyl gallate) were all less effective than α-tocopherol at protecting hydroxylase activity during the 100,000*g* centrifugation. As reported earlier (6), the hydroxylase activity had variable stability. Although about 50% of the activity of the 12,000*g* supernatant was retained after storage at −10 C for 1 month, all of the activity in the 100,000*g* pellet was lost within several h of storage at 0 or −10 C. When the 100,000*g* pellet was washed by resuspending it in fresh grinding medium and then recentrifuging at 100,000*g* for 1 h, all of the activity was lost. When the original 100,000*g* supernatant was added to the washed pellet, the activity was partially restored (50%). The extent of restoration was constant over a span of 10 to 200 μl supernatant added to a constant amount of a pellet suspension.

An oleoyl-CoA:phosphatidylcholine acyltransferase activity was recovered in the 100,000*g* pellet with no activity in the supernatant. Just as with hydroxylase activity, NADH oxidation (in the absence of exogenous substrate) required the combination of both washed pellet and supernatant to regain all the activity which was in the 12,000*g* supernatant. It was not known if the electrons from this high NADH oxidation rate were channeled into oleate hydroxylation, but both microsomal activities exhibited an absolute requirement for a soluble component (5).

Table I. *Stimulation of Microsomal Castor Bean Hydroxylase Activity by Castor Bean Supernatant, Safflower Supernatant, and Purified Catalase*

Enzyme Source	Supernatant, 100,000*g*	Endogenous Catalase	Exogenous Catalase	Ricinoleic Acid Formed
		μmol/min		*nmol/h*
Supernatant, 12,000*g*		12.24		0.33
Supernatant, 100,000*g*		10.74		0.007
Pellet, 100,000*g*				0.096
Washed pellet, 100,000*g*		0.58		0
	Castor bean	11.32		0.16
	Castor bean dialyzed	11.15		0.16
	Castor bean boiled	0		0.03
	Safflower	6.88		0.33
	Safflower dialyzed	7.89		0.34
	Safflower boiled	0		0.04
		0.58	0.2	0
		0.58	0.4	0.059
		0.58	1.0	0.064
		0.58	10	0.089
		0.58	3000	0.094

Stimulation by Safflower Supernatant and Catalase. Although dialysis of the 100,000*g* castor bean supernatant did not affect its capacity to restore activity to the 100,000*g* washed pellet (Table I), boiling the 100,000*g* supernatant did eliminate its ability to restore activity to the 100,000*g* pellet. We then tried to restore activity to the 100,000*g* castor bean washed pellet by adding 100,000*g* supernatant from developing safflower seeds. The safflower supernatant (50 μl) restored all (100%) of the activity to the castor bean pellet, whereas castor bean supernatant (10–200 μl) had only been able to restore about 50% of the activity that had been in the 12,000*g* supernatant. The stimulatory "factor(s)" in the safflower extract was also nondialyzable and heat-sensitive.

Inasmuch as previous workers have shown that liver catalase markedly stimulates liver as well as plant Δ^9-desaturase (1, 2, 7), purified catalase from bovine liver was added to the 100,000*g* washed castor bean pellet and found to restore part of the hydroxylase activity. Ten units of catalase activity was about 50% as effective as castor bean supernatant and about 25% as effective as safflower supernatant at restoring hydroxylase activity to the washed microsomes. Higher concentrations of purified catalase or either of the 100,000*g* supernatants (from castor bean or safflower) did not cause any further stimulation of activity. The levels of endogenous catalase in the various castor bean and safflower fractions are also shown in Table I. Because the safflower supernatant actually has less endogenous catalase activity than does the castor bean supernatant, the enhanced stimulation caused by the safflower supernatant cannot be related solely to catalase activity. These data suggest that there is a nondialysable, heat-sensitive component other than catalase which is required for full hydroxylase activity. It is also possible that catalase may not be required during *in vivo* hydroxylation, but only under *in vitro* experimental conditions. Because catalase activity is associated only with micro-

bodies, the question then arises as to the interaction of catalase in microbodies with the hydroxylase associated with microsomes (endoplasmic reticulum?). Several other types of hydroxylases require catalase for full activity (1). Some of these hydroxylases can be stimulated by boiled catalase or BSA, which suggests a general protein effect independent of catalase activity. Catalase has also been shown to be required for the *in vitro* activity of safflower stearyl-ACP desaturase (personal communication, T. McKeon) and for several mammalian desaturases (2, 7). In one of these systems, exogenous catalase restored all of the activity to the 100,000*g* washed pellet (2), whereas, in others, it restored only part of the activity (7). A soluble lipoprotein with high levels of lysophospholipids has been partially purified from rat liver and shown to be required for full activity of linoleic acid desaturase (5, 8).

Investigation on Nature of Substrate Required for Hydroxylation. In the investigation reported in 1966 (6), the evidence suggested that oleoyl-CoA was the substrate for hydroxylation, with the presumed formation of ricinoleoyl-CoA. A time-course study was therefore performed to determine the substrate-product relation with the present system (Fig. 1). Within the first 2 min, there was a transfer of at least 60% of the labeled oleate from oleoyl-CoA to some form of lipid. After 10 min, there was no further decrease in the amount of oleoyl-CoA. The remaining 10 to 20% of oleoyl-CoA may have been adsorbed to the membrane or to the glass tube. At no time was any ricinoleoyl-CoA detected. Ricinoleic acid was found in the lipid fraction and was synthesized rapidly for the first 10 min. After the initial "burst" of transfer of [^{14}C]oleate to the lipid fraction, the levels of oleate in the lipid fraction slowly decreased for the remainder of the experiment. Inasmuch as this rate of oleate depletion was inversely proportional to the rate of ricinoleate production, it would suggest that the hydroxylation of oleate occurred on a lipid.

Lipids from a time-course experiment similar to that in Figure 1 were separated by TLC (Fig. 2). An immediate decrease in oleoyl-CoA (Fig. 1) was similarly observed (data not shown). After 5 min, most of the [^{14}C]oleate was detected in the PC fraction. The levels of oleate in PC decreased rapidly for the next 15 min and then at a more gradual rate for the remainder of the experiment. In the first 10 min, there was a small, but reproducible, appearance of ricinoleate in the PC fraction. The majority of the ricinoleate produced was found in the nonpolar lipid fraction.

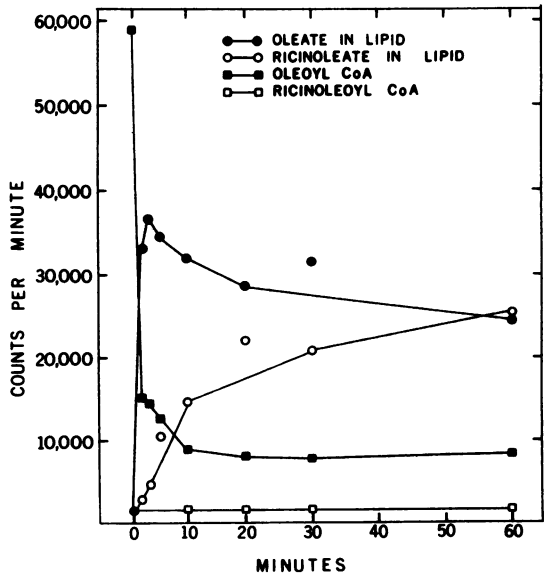

FIG. 1. Time-course study of the amounts of oleoyl-CoA, ricinoleoyl-CoA, and oleate and ricinoleate in the total lipids. Acyl-CoAs were separated from lipids by the Bligh and Dyer extraction procedure (3).

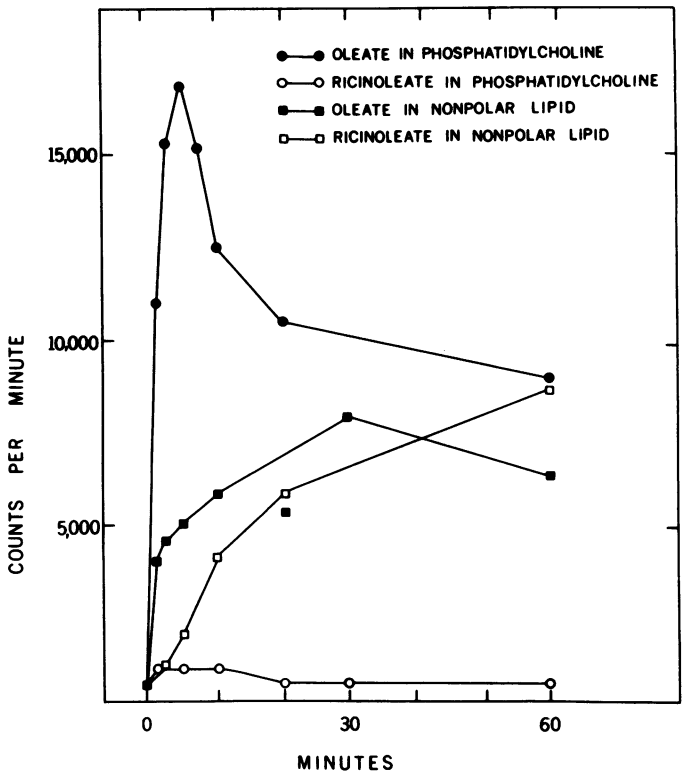

FIG. 2. Time-course study of oleate and ricinoleate in lipid classes. PC was separated from the nonpolar lipids by TLC and oleate and ricinoleate were measured by radio-GLC as described under "Materials and Methods."

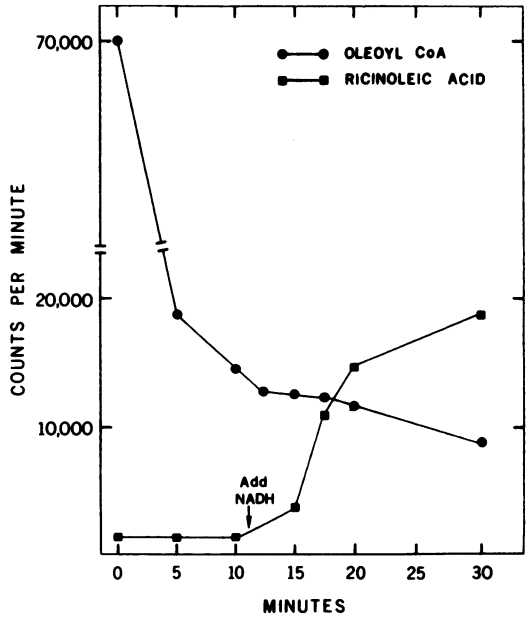

FIG. 3. Effect of delayed addition of NADH on ricinoleic acid formation in the lipid fraction.

Unfortunately, because the Δ^{12}-hydroxy function caused the various glyceride esters of ricinoleic acid to migrate to abnormal rates on TLC, it was not possible to separate accurately the various components of the nonpolar lipid fraction. In the first 10 min, there was an initial rapid appearance of oleate into the nonpolar lipid fraction and it then seemed to level off for the remainder of the experiment. This experiment suggested, however, that the

formation of oleoyl-PC preceded the hydroxylation reaction, especially inasmuch as the rate of oleoyl-PC depletion was inversely proportional to the rate of ricinoleoyl-nonpolar lipid synthesis for the last 55 min of the experiment. Since there was very little buildup of ricinoleoyl-PC, it was not possible to determine whether the hydroxylation occurred on PC or on some nonpolar lipid which was the oleate acceptor from PC. The lack of buildup of ricinoleoyl-PC may also be related either to a high activity of a ricinoleate-specific PC to glyceride acyltransferase or to an endogenous phospholipase A.

In the next experiment (Fig. 3), [^{14}C]oleoyl-CoA was added to the enzyme preparation in the absence of NADH. At the point when most of the oleoyl-CoA had been transferred to lipids (12 min), NADH then was added. An immediate formation of ricinoleic acid occurred upon the addition of NADH. Twenty min after the addition of NADH, about 18,000 cpm ricinoleate accumulated in the lipid fraction but, during the corresponding period, there was a decrease of only about 3500 cpm as oleoyl-CoA. This experiment provided further evidence that the hydroxylation of oleate occurred with a lipid intermediate rather than with oleoyl-CoA. To give further support to the postulate that the hydroxylation of oleate did occur on PC as the lipid intermediate, it would be necessary to explain why the number of counts in oleoyl-CoA never dropped below 10 to 20% (Fig. 1) and why there was very little accumulation of ricinoleoyl-PC (Fig. 2). With the safflower system, the oleate desaturase activity was demonstrated to occur on PC because all the oleoyl-CoA was depleted during the experiment with a simultaneous buildup of linoleoyl-PC (15).

In another experiment 2-[1-^{14}C]oleoyl-labeled PC was tested as a substrate. When 3.5 nmol [^{14}C]oleoyl-PC (in 25 μl ethanol) was incubated with 12,000g supernatant and 1 mM NADH, only 0.3 nmol oleate was hydroxylated after 60 min. When the same amount of [^{14}C]oleoyl-CoA was similarly incubated, 2.1 nmol oleate were hydroxylated. This low rate of hydroxylation of exogenous oleoyl-PC is comparable to the rates of its desaturation with developing safflower extracts (13). It is of course possible that, when oleoyl-PC is added in ethanol, it may not be able to interact with the active site of the hydroxylase. If the exogenous [^{14}C]oleoyl-PC can somehow be incorporated into the boundary lipids of the membrane which contain the hydroxylase, higher rates of hydroxylation will then be observed. In spite of the low rates of hydroxylation of exogenous [^{14}C]oleoyl-PC, the observation that there was some measurable activity still suggests that PC may be the substrate of hydroxylation.

To determine the position of [^{14}C]oleate in the [^{14}C]PC, [^{14}C]-oleoyl-CoA was incubated for 5 min with the 12,000g supernatant extract. After 5 min, 61% of the labeled lipid was PC (Table II). Phospholipase A_2 was then added to the reaction mixture and it was incubated for another 10 min. After phospholipase treatment, 91% of the counts were in the free fatty acid fraction, and no new counts were found in lyso-PC. This experiment shows that virtually all of the labeled oleate on PC was at carbon 2 of the glycerol moiety and confirms the observation of Vijay and Stumpf (17) that the oleoyl moiety of oleoyl-CoA is inserted primarily in position 2 of PC. This result suggests that the glycerol moiety of PC and the acyl moiety on its number 1 carbon were not channeled into triacylglycerol synthesis. Instead, there may be a pool of PC which is constantly acylated (perhaps hydroxylated) and then deacylated at its carbon 2. We also found that lyso-PC represented 16% of all the phospholipids in the 12,000g supernatant (data not shown), which may represent a large number of "receptor" sites for an acylation-hydroxylation-deacylation mechanism. The addition of 10–50 μM exogenous lyso-PC, phosphatidic acid, or monoolein, however, inhibited the hydroxylase. The addition of the same concentration of 1,2-diolein, glycerol 3-P, CDP choline, choline, and D- or L-carnitine had no effect on hydroxylase activity.

Table II. *Phospholipase A_2 Treatment of Oleoyl-CoA "Charged" Microsomes*

The reaction mixture contained the standard components described under "Materials and Methods" (12,000g supernatant was used as a source of enzyme). After incubation for 5 min, 0.05 mg phospholipase A_2 from *Crotalus durissus terrificus* venom and 20 μmol $CaCl_2$ were added. The phospholipase was allowed to incubate with the mixture for 10 min. Lipids then were extracted with isopropanol-petroleum ether, separated on the polar TLC system, and scraped and counted as described.

Treatment	Lipid Class					
	Lyso-PC		PC		Free Fatty Acid	
		Labeled lipid		Labeled lipid		Labeled lipid
	cpm	*%*	*cpm*	*%*	*cpm*	*%*
Control	905	4	16,885	61	9,898	33
Phospholipase-treated	973	4	1,290	5	24,051	91

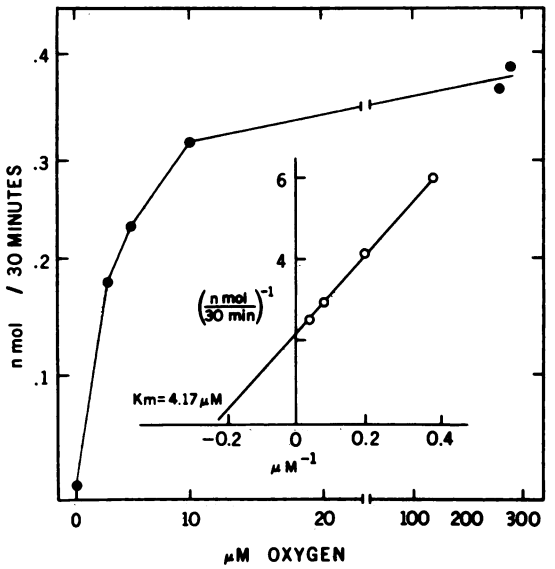

FIG. 4. Effect of various concentrations of molecular O_2 on the activity of oleate hydroxylase. The Lineweaver-Burk plot was used to calculate an apparent K_m of 4.17 μM for O_2.

Other Enzymic Properties. The apparent hydroxylase activity was not saturated by any concentration of oleoyl-CoA. The activity continued to increase at a proportional rate up to 200 μM oleoyl-CoA. These unusual kinetics made it impossible to determine whether *in vitro* rates of ricinoleic acid biosynthesis were sufficient to account for *in vivo* rates of synthesis during seed development. Inasmuch as our data (Figs. 1–3) suggested that the actual hydroxylation did not occur on oleoyl-CoA, the unusual kinetics may be due to the one or more acyl transfer reactions that preceded hydroxylation.

Normal saturation kinetics were observed with varying concentrations of O_2 (Fig. 4). The Lineweaver-Burk plot was used to calculate an apparent K_m of 4.17 μM for O_2.

Carbon monoxide and metyrapone, both inhibitors of Cyt P450, had no effect on the hydroxylase activity (Table III). These results would tend to exclude Cyt P450 as a component of the hydroxylase reaction. Although it was previously reported that the hydroxylase activity was inhibited by KCN (6), we were unable to observe any cyanide inhibition with the 12,000g supernatant and only a slight inhibition with the 100,000g pellet plus purified catalase. The catalase activity in the reaction mixtures was drastically inhibited

Table III. *Effect of Various Inhibitors on Castor Bean Hydroxylase*

Experiment	Treatment	Ricinoleic Acid Formed	Endogenous Catalase Activity
		nmol/h	*IU*
1	20% O_2 + 80% N_2	0.31	
	20% O_2 + 80% CO	0.32	
	1% O_2 + 99% CO	0.30	
	Air + 1 mM metyrapone	0.32	
2	Control (12,000*g* supernatant)	0.28	1.92
	10 μM KCN (pH 7)	0.26	0.85
	100 μM KCN	0.27	0.115
	1,000 μM KCN	0.27	0.011
	Pellet 100,000*g* + purified catalase	0.31	0.95
	10 μM KCN	0.25	0.58
	100 μM KCN	0.26	0.13
	1,000 μM KCN	0.26	0.028

by KCN, but there was little or no corresponding decrease in hydroxylase activity. This experiment suggests that catalase may be serving some function in the hydroxylase system other than just removing H_2O_2.

The addition of ATP, CoA, ACP, ferredoxin, and ferredoxin reductase had no effect on hydroxylase activity. In contrast to previously reported results (6), a 50% inhibition of activity by $FeSO_4$ and a slight stimulation of activity by EDTA were observed.

In conclusion, this investigation was of interest for a number of reasons. First, the lack of inhibition by high concentrations of both CO (99%) and cyanide (1 mM) suggested that the hydroxylation reaction did not involve either a Cyt P450 or a cyanide-sensitive iron porphyrin system.

Second, the data strongly suggested that oleoyl-CoA was not the immediate substrate for hydroxylation. Although oleoyl-PC was rapidly formed and utilized, the low levels of ricinoleoyl-PC formed throughout the period of reaction would add caution to the conclusion that an oleoyl-PC substrate to a ricinoleoyl-PC product was involved in the actual hydroxylation mechanism. The identification of the ricinoleoyl-nonpolar lipid product would be pertinent to the resolution of this problem.

Third, whereas the hydroxylase activity in the 12,000*g* supernatant of developing castor bean is relatively stable, there is a rapid loss of activity after subsequent centrifugation (100,000*g* for 1 h). Some activity can be restored to the microsomes by addition of catalase or other soluble heat-sensitive components. The nature of the cofactor requirements and instability of the 100,000*g* pellet require further study.

Finally, the similarities between oleate hydroxylation in castor bean and oleate desaturation in safflower and other seeds were quite striking and may have some evolutionary importance. Both activities were CO-insensitive and preferred NADH as an electron donor (14, 17). Like the hydroxylase, the safflower desaturase was also microsomal (17), but the catalase and supernatant requirements have not been investigated with the latter system.

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